Effects of lithium and valproate on amphetamine-induced oxidative stress generation in an animal model of mania

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Objective: Previous studies have suggested that oxidative stress may play a role in the pathophysiology of bipolar disorder (BD). Moreover, recent studies indicate that lithium and valproate exert neuroprotective effects against oxidative stress. We studied the effects of the mood stabilizers lithium and valproate on amphetamine-induced oxidative stress in an animal model of mania.

Methods: In the first model (reversal treatment), adult male Wistar rats received d-amphetamine or saline for 14 days, and between the 8th and 14th days, they were treated with lithium, valproate or saline. In the second model (prevention treatment), rats were pretreated with lithium, valproate or saline, and between the 8th and 14th days, they received d-amphetamine or saline. We assessed locomotor activity with the open-field task. We measured thiobarbituric acid reactive substances (TBARS) and protein carbonyl formation, as parameters of oxidative stress, and superoxide dismutase (SOD) and catalase (CAT), the major antioxidant enzymes, in the prefrontal cortex and hippocampus.

Results: Lithium and valproate reversed (reversal treatment model) and prevented (prevention treatment model) amphetamine-induced hyperactivity and reversed and prevented amphetamine-induced TBARS formation in both experiments. However, the co-administration of lithium or valproate with amphetamine increased lipid peroxidation, depending on the brain region and treatment regimen. No changes in protein carbonyl formation were observed. SOD activity varied with different treatment regimens, and CAT activity increased when the index of lipid peroxidation was more robust.

Conclusion: Our findings suggest that lithium and valproate exert protective effects against amphetamine-induced oxidative stress in vivo, further supporting the hypothesis that oxidative stress may be associated with the pathophysiology of BD.

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Introduction

Recent studies have consistently reported increased products of lipid peroxidation and alterations of the major antioxidant enzymes in people with bipolar disorder (BD). It has been widely demonstrated that the generation of reactive oxygen species (ROS) plays a critical role in the pathophysiology of several neuropsychiatric disorders. The brain is particularly vulnerable to ROS production because it metabolizes 20% of total body oxygen and has a limited amount of antioxidant capacity. In situations where the generation of free radicals exceeds the capacity of antioxidant defence, oxidative stress may lead to membrane degradation, cellular dysfunction and apoptosis. This might be relevant for the pathogenesis of BD, because in-vivo magnetic resonance spectroscopy studies have demonstrated changes in brain compounds related to oxidative phosphorylation, energy production and phospholipid metabolism. In addition, it has been hypothesized that BD is associated with mitochondrial dysfunction, and abnormalities in respiratory complex activity and energy production may lead to cellular degeneration.

Additional associations between oxidative and antioxidative systems in BD have been demonstrated in pharmacological studies. In primary cultured neuronal cells, lithium and valproate (first-line mood stabilizers) prevent glutamate-induced oxidative stress and increase mRNA and protein levels of glutathione S-transferase M1 isoenzyme and valproate inhibits FeCl₂-induced lipid peroxidation and protein oxidation. More recently, King and Jope showed that lithium protects against caspase activation that is induced by intrinsic and extrinsic sources of oxidative stress in human neuroblastoma SH-SY5Y cells. Together, these findings strongly suggest that the modulation of ROS generation may be relevant in the mood-stabilizing effects of lithium and valproate; however, the effects of lithium and valproate on oxidative stress have not been studied in vivo. We recently found that repeated amphetamine exposure increases thiobarbituric acid-reactive species and protein carbonyl formation in the rat hippocampus and cerebral cortex, 2 brain regions related to mood regulation. In addition, recent genetic and postmortem studies suggest that BD may be associated with altered dopaminergic transmission. Because of these findings, we designed the present study to investigate the effects of lithium and valproate on lipid and protein oxidation levels (markers of oxidative stress) and on superoxide dismutase (SOD) and catalase (CAT) activities (the major antioxidant enzymes) in a dopaminergic model of mania.

Methods

We conducted the study, using adult male Wistar rats obtained from our breeding colony. The animals were housed 5 to a cage, on a 12-hour light/dark cycle (lights on at 7:00 am), with free access to food and water. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behaviour (SBNeC) recommendations for animal care. This study was approved by the local ethics committee (Comitê de Ética em Pesquisa da Universidade do Extremo Sul Catarinense).

Reversal treatment

In this model, we reproduced the treatment of an acute manic episode. Rats received either a daily injection of d-amphetamine, 2 mg/kg (Sigma, St. Louis, Mo.), or saline for 14 days. Between the 8th and the 14th days, the animals were divided into 3 experimental groups (12–15 animals per group): 1 group received lithium, 47.5 mg/kg intraperitoneal (IP), twice a day; the second group received valproate, 200 mg/kg IP, twice a day; and the third group received saline IP twice a day. Locomotor activity was assessed 2 hours after the last injection, and rats were sacrificed by decapitation immediately following the open-field task. The prefrontal cortex and hippocampus were dissected, rapidly frozen and stored at –80°C until assayed.

Prevention treatment

In this model, we reproduced the maintenance treatment of BD. Rats received either lithium, 47.5 mg/kg; valproate, 200 mg/kg; or saline IP twice a day for 14 days. The animals were then divided into 2 groups (12–15 animals per group). Between the 8th and the 14th days, each group received one daily IP injection of d-amphetamine, 2 mg/kg, or saline. Locomotor activity was assessed 2 hours after the last injection,
and rats were sacrificed by decapitation immediately following the open-field task. The prefrontal cortex and hippocampus were dissected, rapidly frozen and stored at –80°C until assayed. All lithium-treated animals presented lithium levels between 0.6–1.2 mEq/L in plasma, as recommended in the treatment of patients with BD.

**Locomotor activity**

We used the open-field task to assess locomotor activity. The task was performed in a 40 × 60 cm open field surrounded by 50 cm-high walls made of brown plywood with a frontal glass wall. The floor of the open field was divided into 12 equal rectangles by black lines. The animals were gently placed on the left rear rectangle and were allowed to explore the arena. Crossings of the black lines and rearings were counted for 5 minutes.

**Oxidative stress parameters**

To determine oxidative damage, we measured the formation of thiobarbituric acid reactive species (TBARS) during an acid-heating reaction, as previously described. The samples were mixed with 1 mL of trichloroacetic acid (TCA) 10% and 1 mL of thiobarbituric acid (TBA) 0.67% and were then heated in a boiling water bath for 15 minutes. TBARS were determined by the absorbance at 535 nm. Oxidative damage to proteins was measured by the quantification of carbonyl groups based on the reaction with dinitrophenylhydrazine (DNPH), as previously described. Proteins were precipitated by the addition of 20% trichloroacetic acid and were re-dissolved in DNPH; the absorbance was read at 370 nm.

To determine CAT activity, the brain tissue was sonicated in 50 mmol/L phosphate buffer (pH 7.0), and the resulting suspension was centrifuged at 3000 g for 10 minutes. The supernatant was used for enzyme assay. CAT activity was measured by the rate of decrease in hydrogen peroxide absorbance at 240 nm. SOD activity was assayed by measuring the inhibition of adrenaline auto-oxidation, as previously described. All biochemical measures were normalized to the protein content, with bovine albumin as standard. All data are presented as mean and standard error of the mean. Differences among the experimental groups evaluating exploratory behaviour were determined by 1-way analysis of variance (ANOVA), followed by the Tukey post hoc test. Biochemical data were analyzed by 1-way ANOVA, and multiple comparisons were performed with the Newman-Keuls test. In all comparisons, statistical significance was set at p < 0.05.

**Results**

**Behaviour**

In the reversal and prevention models, amphetamine increased locomotor and rearing behaviours, and the administration of lithium and valproate reversed and prevented amphetamine-induced hyperactivity (Fig. 1, Fig. 2). Lithium and valproate did not affect behavioural measures in animals treated with saline, suggesting that the effects of mood stabilizers on animals treated with amphetamine

**Fig. 1:** Numbers of crossings (A) and rearings (B) in the reversal model (n = 12 for each group). Bars represent means; error bars represent standard error of the means (SEM). Crossings = 1-way analysis of variance (ANOVA); F<sub>5,66</sub> = 22.68; p < 0.001; *different from all groups (Tukey’s post hoc; p < 0.001). Rearings = 1-way ANOVA; F<sub>5,66</sub> = 15.28; p < 0.001; *different from all groups (Tukey’s post hoc; p < 0.001). Rats were pretreated with amphetamine (Amph) for 7 days and then treated with Amph plus mood stabilizers between the 8th and the 14th days. Li = lithium, Sal = saline, VPA = valproate.
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were not related to sedation. These results replicate our recent findings and confirm the reproducibility of this behavioural model.

Oxidative stress parameters

Reversal treatment

Amphetamine increased TBARS levels in the prefrontal cortex, and this effect was reversed by both mood stabilizers (Fig. 3a). The administration of lithium and valproate

Fig. 2: Numbers of crossings (A) and rearings (B) in the prevention model (n = 12 for each group). Bars represent means; error bars represent standard error of the means (SEM). Crossings = 1-way analysis of variance (ANOVA); F_{5,66} = 7.04; p < 0.001; *different from all groups (Tukey’s post hoc; p < 0.001 from Sal + Sal and Li + Sal; p < 0.001 from VPA + Sal; p = 0.018 from Li + Amph; p = 0.023 from VPA + Amph). Rearings = 1-way ANOVA; F_{5,66} = 4.80; p < 0.001; *different from all groups (Tukey’s post hoc; p < 0.001 from Sal + Sal and VPA + Sal; p = 0.006 from Li + Sal and VPA + Amph; p = 0.022 from Li + Amph). Rats were pretreated with mood stabilizers for 7 days and then treated with mood stabilizers plus amphetamine (Amph) between the 8th and the 14th days. Sal = saline, VPA = valproate.

Fig. 3: TBARS levels in the prefrontal cortex and hippocampus after reversal (A) and prevention (B) treatments (n = 5 for each group). Prefrontal cortex in the reversal treatment group = 1-way analysis of variance (ANOVA); F_{4,20} = 3.14; p = 0.02. Hippocampus in the reversal treatment group = 1-way ANOVA; F_{4,20} = 22.44; p < 0.001. Prefrontal cortex in the prevention treatment group = 1-way ANOVA; F_{4,20} = 5.34; p = 0.001. Hippocampus in the prevention treatment group = 1-way ANOVA; F_{4,20} = 15.66; p < 0.001. Bars represent means; error bars represent standard error of the means (SEM); *different from the Sal + Sal group (Newman-Keuls post hoc; p < 0.05); # different from the Amph + Sal group (Newman-Keuls post hoc; p < 0.05). Amph = amphetamine, Li = lithium, MDA = malondialdehyde, Sal = saline, TBARS = thiobarbituric acid reactive species.
increased TBARS formation in the hippocampus of rats pre-treated with amphetamine. No changes were observed in protein carbonyl formation (data not shown).

**Prevention treatment**

Amphetamine increased TBARS levels in the prefrontal cortex and hippocampus, and these effects were prevented by valproate pretreatment (Fig. 3b). Lithium pretreatment partially prevented amphetamine-induced lipid peroxidation in the rat hippocampus but augmented amphetamine-induced lipid peroxidation in the prefrontal cortex. As in the reversal treatment, no changes were observed in protein carbonyl formation (data not shown). Taken together, our findings suggest that the neuroprotective effects of lithium and valproate on amphetamine-induced oxidative stress vary with brain region and treatment regimen. When interpreting the results, it...
is worth noting that the administration of lithium and valproate alone had no effect on oxidative stress.

Antioxidant enzyme activity

Reversal treatment

In this model, valproate increased SOD levels in the prefrontal cortex, whereas lithium, valproate and lithium plus amphetamine decreased SOD in the rat hippocampus (Fig. 4a). No changes were observed on CAT activity (Fig. 5a).

Prevention treatment

Lithium, valproate, lithium plus amphetamine and valproate plus amphetamine decreased SOD levels in the prefrontal cortex, whereas valproate increased SOD in the hippocampus (Fig. 4b). Amphetamine increased CAT levels, whereas lithium plus amphetamine, and valproate plus amphetamine decreased CAT levels in the rat hippocampus (Fig. 5b).

Discussion

In this study, we demonstrated that lithium and valproate reversed amphetamine-induced lipid peroxidation in the prefrontal cortex and prevented amphetamine-induced lipid peroxidation in the hippocampus. Using the present model, we were able to reproduce previous findings of the neuroprotective effects of mood stabilizers in response to oxidative stress.10–12 Our results are consistent with existing evidence that mood stabilizers share significant neuroprotective properties.24 However, we also found that the co-administration of lithium or valproate with amphetamine can increase TBARS formation, suggesting that their effects on oxidative stress vary depending on the brain region and treatment regimen. Because the administration of lithium or valproate alone did not induce oxidative damage, it is conceivable that they might augment amphetamine-induced oxidative stress in some situations. Amphetamine can enhance ROS formation through several pathways, for example, through autoxidation of dopamine with the formation of highly reactive quinones,25 direct inhibition of mitochondrial electron transport chain complexes26 and increased glutamate release.27 Although the mechanisms by which mood stabilizers decrease ROS generation are poorly understood, it has been suggested that they might involve the induction of the molecular chaperone GRP78,28 buffering [Ca2+]i levels,29 and by stabilizing mitochondrial enzymes.24 However, we also found that the co-administration of lithium or valproate with amphetamine can increase TBARS formation, suggesting that their effects on oxidative stress vary depending on the brain region and treatment regimen.

In conclusion, we have demonstrated that lithium and valproate can reverse and prevent amphetamine-induced oxidative stress in vivo. Our findings further support the notion that neuroprotection may be one of the mechanisms of action of mood stabilizers and that oxidative stress may play a role in the pathophysiology of BD.

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Contributors: Drs. Frey, Kapczinski and Quevedo and Mr. Martins designed the study. Mses. Valvassori, Réus, Petronilho and Bardini and Dr. Dal-Pizzol acquired and analyzed the data. Drs. Frey, Dal-Pizzol and Kapczinski and Mr. Martins wrote the article; Drs. Frey and Quevedo and Mses. Valvassori, Réus, Petronilho and Bardini critically reviewed it. All authors gave final approval for publication.

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